Free Rad. Res., Vol 34, pp. 477-484
Reprints available directly from the publisher
Photocopying permitted by license only

Lipid Peroxidation is Increased in Paraoxonase L55 Homozygotes Compared with M-Allele Carriers

RIIKKA MALIN[a], SEPPO LAINE[b], VAPPU RANTALAIHO[c], OLE WIRTA[c], AMOS PASTERNACK[c], HANNU JOKELA[a], HANNU ALHO[d,e], TIMO KOIVULA[a] and TERHO LEHTIMÄKI[a,*]

[a]*Tampere University Hospital, Centre for Laboratory Medicine, Department of Clinical Chemistry and University of Tampere, Medical School,* [b]*Tampere University Hospital, Department of Microbiology and* [c]*Department of Internal Medicine, P.O. Box 2000, 33521 Tampere,* [d]*National Public Health institute, Department of Mental Health and Alcohol Research, P.O. Box 719, 00101 Helsinki and* [e]*Research Unit of Alcohol Diseases, University of Helsinki, Helsinki, Finland*

Accepted for publication by Prof. B. Halliwell

(Received 24 August 2000)

Human serum paraoxonase (PON) is an antioxidative enzyme, which circulates on high-density lipoproteins and appears to use oxidized phospholipids as physiological substrates. PON M/L55 substitution changes the ability of PON to prevent lipid oxidation. Urinary 8-iso-$PGF_{2\alpha}$ (one of F_2-isoprostanes) may represent a non-invasive *in vivo* index of free radical generation and we propose that PON might influence the biosynthesis of 8-iso-$PGF_{2\alpha}$ in the vasculature. We studied the urinary excretion of 8-iso-$PGF_{2\alpha}$ and related it to PON M/L55 genotypes in patients with type 2 diabetes mellitus (n = 55) and non-diabetic control subjects (n = 55). Urinary 8-iso-$PGF_{2\alpha}$was determined by competitive ELISA and the PON genotype by a PCR based restriction enzyme digestion method. LL homozygotes were compared to M-allele carriers (ML heterozygotes and MM homozygotes). The urinary excretion of 8-iso-$PGF_{2\alpha}$ among non-diabetic non-smoking LL homozygotes was 3995.5 ± 3352.8 ng/24-hour and among M-allele carriers 1689.8 ± 1051.3 ng/24-hour ($p = 0.017$, ANCOVA; gender, hypertension, total cholesterol, triglycerides and LDL cholesterol as covariates). The excretion of 8-iso-$PGF_{2\alpha}$, was increased in type 2 diabetes mellitus compared to non-diabetic control subjects. PON may thus protect against oxidative stress by destroying some biologically active lipids. Excretion of 8-iso-$PGF_{2\alpha}$ is increased in type 2 diabetes, which may reflect oxidant injury.

Keywords: Paraoxonase, Lipid peroxide, 8-iso-$PGF_{2\alpha}$, Diabetes mellitus, type 2

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) in the artery walls is one of the most important factors involved in the initiation of atherosclerosis. High-density lipoprotein (HDL) retards the accumulation of lipid peroxides on LDL particles when incubated under oxidizing conditions *in vitro*.[1] This process is apparently due to paraoxonase (PON), an enzyme associated with HDL. PON has two functions: First, it has been shown to contribute to the detoxification of organophosphorous compounds, including the pesticide paraoxon, and, secondly, it can hydrolyze lipid peroxides and thus protect LDL from oxidation.[2]

The PON gene (*PON1*) has two common genetic polymorphisms giving rise to amino acid

* Corresponding author. Terho Lehtimäki, MD, PhD. Tampere University Hospital, Laboratory of Atherosclerosis Genetics. FinnMedi 2, P.O. Box 2000. FIN-33521 Tampere, Finland. Tel: +358 3 247 4066. Fax: +358 3 247 4168. E-mail: bltele@uta.fi

substitutions of methionine (M) for leucine (L) at position 55 and arginine (R) for glutamine (Q) at position 192 of the protein. These polymorphisms are in strong linkage disequilibrium that favors the simultaneous presence of the L- and R-alleles. Both M/L55 and R/Q192 substitutions have been shown to affect serum PON activity. PON activity must be determined against paraoxon because the physiological substrate of PON is unknown.[3] When considering the M/L55 polymorphism, LL homozygotes have the highest activity towards paraoxon followed by ML heterozygotes and MM homozygotes with the lowest activity. This stands in opposite sequence to the specificity of PON isoenzymes with regard to hydrolysis of lipid peroxides[2] and has led to a hypothesis that there is yet another substrate for PON, the product of which modulates atherogenesis.

The discovery of new markers of oxidative stress has improved our ability to measure oxidant injury. These markers include F_2-isoprostanes, which are a family of prostaglandin isomers. They are initially formed *in situ* on phospholipids and then released, presumably by phospholipases.[4] F_2-isoprostanes are derived from arachidonic acid, which undergoes peroxidation by a mechanism catalyzed by free radicals and unrelated to cyclooxygenase to yield arachidonyl radical intermediates, which are then transformed to a series of prostaglandin F_2-like compounds.[5] These compounds circulate in plasma and finally are excreted into urine.[6] 8-iso-$PGF_{2\alpha}$, which is one of the most abundantly formed compounds following peroxidation of arachidonic acid, is a potent renal[7] and pulmonary vessel[8]vasoconstrictor in animal models. 8-iso-$PGF_{2\alpha}$ is also the cause of platelet shape change but not aggregation.[9] Increased plasma levels of F_2-isoprostanes occur in smokers,[10] patients with the hepatorenal syndrome,[11] and in patients with type 2 (non-insulin dependent) diabetes mellitus.[12] The levels of F_2-isoprostanes, both free in the circulation and esterified to tissue phospholipids, increase also dramatically in experimental animals subject to oxidant injury[4] and in patients with acute myocardial infarction.[13] This suggests that urinary 8-iso-$PGF_{2\alpha}$ could be a useful non-invasive *in vivo* index of free radical generation.

We hypothesize that PON, as an antioxidative enzyme, might influence the biosynthesis of 8-iso-$PGF_{2\alpha}$ in the vasculature. To measure the actual rate of 8-iso-$PGF_{2\alpha}$ synthesis *in vivo*, we studied the urinary excretion of this oxidative stress marker and related the rate of excretion to PON M/L55 genotypes. In addition, we studied if the excretion of 8-iso-$PGF_{2\alpha}$ is increased in type 2 diabetes or among smokers.

MATERIALS AND METHODS

Patients and Control Subjects

One hundred and fifty recently diagnosed type 2 diabetes outpatients at the municipal health care center of the city of Tampere and the same number of non-diabetic control subjects attending the same facilities and matched for age and gender, were originally recruited between 1985 and 1988. The diabetic patients fulfilled the diagnostic criteria of the WHO for type 2 diabetes. Subjects with a serious disease or a shortened life expectancy (e.g., cancer or cirrhose) were excluded from both study groups. The recruitment of patients and control subjects has been previously discussed in detail.[14]

A group of non-diabetic control subjects ($n = 55$) and patients with type 2 diabetes ($n = 55$) were randomly chosen for this study of those subjects that were re-evaluated after a mean of nine years of disease duration and follow-up, respectively. At the time of re-evaluation, those control subjects who had an elevated fasting blood glucose level were excluded. Weight and height were recorded and the body mass index (BMI) was calculated (kg/m^2). Blood

pressure was measured as recommended by the American Heart Association.[15] A subject was considered to have hypertension if he or she was on antihypertensive medication, or if the blood pressure was greater than 160/95 mmHg. At the time of this study, 25% of the diabetic participants and 15% of the control subjects used ACE inhibitors. The rate of diuretic use was 16% and 13% and of betablockers 33% and 31%, respectively (p = NS, diabetic vs. control subjects). Diabetic subjects were treated by diet only, in combination with insulin or with an oral antidiabetic therapy and they received dietary counselling to reduce their intake of fat and total energy. All study participants gave written informed consent to the study. The study was approved by the ethics committee of the Tampere University Hospital.

Biochemical Assays

All blood samples were drawn after the subjects had had a 12-hour overnight fast. Serum total cholesterol and triglycerides were measured by the dry slide technique (Ektachem 700 analyzer, Johnson and Johnson Clinical Diagnostics, Rochester, NY, USA). The HDL concentration was determined after precipitation of LDL using the same technique. The LDL concentration was calculated by Friedewald's formula.[16] The glomerular filtration rate (GFR) was measured by the [^{51}Cr]EDTA plasma clearance technique.[17]The concentration of ascorbic acid was measured by high-performance liquid chromatography (HPLC) with an electrochemical detector.[18] Plasma α-tocopherol was measured by a modified HPLC method[19] with a LC-amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). The protein thiol groups were determined as described earlier.[20]

Each subject collected the urine for 24-hours. The total volume was mixed and stored frozen at –70°C until analyzed. Thawed urine samples were centrifuged at 10 000 × g for 10 min, and, after proper dilution, the supernatant was used for the determination of 8-iso-$PGF_{2\alpha}$ by a competitive ELISA according to manufacturer's instructions (R & D Systems Inc., Minneapolis, MN, USA). The manufacturer has established the specificity of the polyclonal antibody used in the kit. The sensitivity of the 8-iso-$PGF_{2\alpha}$ assay is typically less than 12.3 pg/ml. Urinary 8-iso-$PGF_{2\alpha}$ was expressed as the total amount excreted in 24 hours. The urinary 8-iso-$PGF_{2\alpha}$/GFR ratio was alternatively used in analysis.

DNA Extraction and PON Genotyping

DNA was isolated from lymphocytes with a commercial kit (Qiagen Inc, Valencia, CA, USA). PON M/L55 genotypes were determined by polymerase chain reaction and restriction enzyme *Hsp92*II (Promega, Madison, WI, USA) digestion as described earlier.[21] The amplification cycle was performed in a PTC-225 thermal cycler (MJ Research Inc., Watertown, MA, USA). After initial denaturation at 96°C for 2.5 min, the DNA was amplified by 40 cycles in the following conditions: denaturation at 96°C for 30 s, annealing at 61°C for 1 min and extension at 72°C for 1 min. *Hsp92*II enzyme digestion followed the instructions of the manufacturer. Digested fragments were separated by electrophoresis on 2.5% agarose gel and visualized with ultraviolet light after ethidium bromide staining.

Statistical Analyses

Because the number of MM homozygotes was small, these subjects were combined for analysis with ML heterozygotes to get two groups: M-allele carriers and LL homozygotes. Discontinuous variables were compared using the χ^2 test and the means of continuous variables using the t-test for independent samples. The differences in the mean urinary excretion of 8-iso-$PGF_{2\alpha}$ between the subgroups were compared statistically after logarithmical transfor-

mation with analysis of covariance (ANCOVA) were gender, hypertension (yes/no), total cholesterol, triglycerides and LDL cholesterol were used as covariates (selection of covariates was based on the variables presented in Table I). The results are expressed as crude data. Data are presented as mean ± standard deviation (SD). A p-value of less than 0.05 was considered to be statistically significant. The computations were made on Statistica for Windows version 5.0 (Statsoft Inc., Tulsa, OK, USA).

RESULTS

Table I summarizes the characteristics of all study subjects by diabetes status. As expected, there were some statistically significant differences between diabetic and control subjects (Table I). The frequencies of the L- and M-alleles of the PON gene were 61% and 39% in control subjects and 63% and 37% in the diabetes patients, respectively (p = NS, control vs. diabetic subjects). The frequency distribution of the genotypes was in Hardy-Weinberg equilibrium. There were no statistically significant differences between the LL homozygotes and M-allele carriers in either diabetic or control subjects for most of the variables presented in Table I. However, in the group of non-diabetic subjects the mean GFR among the LL homozygotes was 94.9 ± 23.7 ml/min/1.73 m^2 and among the carriers of the M-allele 82.8 ± 12.1 ml/min/1.73 m^2 (p = 0.017, t-test). Among the control subjects, the male/female-ratio was 12 / 5 in the group of LL homozygotes and 14 / 24 in the group of M-allele carriers (p = 0.021, χ^2 test). Also, the HDL cholesterol was lower among control LL homozygotes (1.0 ± 0.3 mmol/l) than among control M-allele carriers (1.3 ± 0.4 mmol/l, p = 0.009, t-test).

TABLE I Characteristics of study participants

	Control subjects (n = 55)	*Diabetic subjects (n = 55)*	*p-value*
Age (years)	57.2 ± 6.1	56.1 ± 7.7	NS
Gender (male/female)[a]	26 / 29	27 / 28	NS
Body mass index (kg/m^2)	29.0 ± 5.0	29.8 ± 5.2	NS
Glomerular filtration rate (ml/min/1.73m^2)	86.4 ± 17.2	100.0 ± 25.0	0.002
Smoking (present/no)[a]	6 / 49	6 / 49	NS
Hypertension (yes/no)[a]	32 / 23	42 / 13	0.042
Glutathione (μmol/l)	544 ± 75	554 ± 89	NS
α-tocopherol (μmol/l)	30.8 ± 7.3	31.1 ± 8.9	NS
Ascorbic acid (μmol/l)	48.2 ± 18.2	46.8 ± 15.6	NS
Total cholesterol (mmol/l)	5.8 ± 1.2	5.3 ± 1.0	0.011
Triglycerides (mmol/l)	1.5 ± 0.7	2.0 ± 1.1	0.003
LDL cholesterol (mmol/l)	3.9 ± 1.1	3.3 ± 0.9	0.001
HDL cholesterol (mmol/l)	1.2 ± 0.4	1.1 ± 0.3	NS

NS = not statistically significant difference.

a. p-values: t-test for independent samples or χ^2 test.

TABLE II 24-hour urinary excretion of 8-iso-$PGF_{2\alpha}$ and the ratio of 8-iso-$PGF_{2\alpha}$ to GFR among diabetic and control subjects by smoking status

	Smoking	*n*	*24-h 8-iso-$PGF_{2\alpha}$ (ng/24-hour)*[a]	*n*	*8-iso-$PGF_{2\alpha}$/GFR (ng/(ml/min/1.73m^2))*[b]
Diabetic subjects					
	Yes	6	4068.8 ± 3703.8	6	45.4 ± 50.5
	No	49	4677.5 ± 4218.0	48	46.9 ± 38.4
Control subjects					
	Yes	6	3195.5 ± 4629.9	6	33.0 ± 48.4
	No	49	2395.6 ± 2278.4	47	29.5 ± 27.1

GFR = glomerular filtration rate.
ANCOVA; gender, hypertension (yes/no), total cholesterol, triglycerides and LDL cholesterol as covariates. Data was transformed logarithmically before analysis.
Non-smoking diabetic vs. non-smoking control subjects: [a]$p < 0.001$; [b]$p = 0.012$.
All diabetic vs. all control subjects: [a]$p = 0.002$; [b]$p = 0.020$.

TABLE III 24-hour urinary excretion of 8-iso-$PGF_{2\alpha}$ and the ratio of 8-iso-$PGF_{2\alpha}$ to GFR among non-smoking diabetic and control subjects by PON M/L55 genotype

	n	*LL*	*n*	*M-allele carriers*
24-h 8-iso-$PGF_{2\alpha}$ (ng/24-hour)				
Diabetic subjects	17	5488.3 ± 4892.1	32	4246.8 ± 3826.7
Control subjects[a]	15	3995.5 ± 3352.8	34	1689.8 ± 1051.3
8-iso-$PGF_{2\alpha}$/GFR (ng/(ml/min/1.73m^2))				
Diabetic subjects	17	51.3 ± 42.3	31	44.5 ± 36.6
Control subjects[b]	14	49.3 ± 39.6	33	21.0 ± 13.2

GFR = glomerular filtration rate.
ANCOVA; gender, hypertension (yes/no), total cholesterol, triglycerides and LDL cholesterol as covariates. Data was transformed logarithmically before analysis.
Non-diabetic LL homozygotes'vs. non-diabetic M-allele carriers: [a]$p = 0.017$, [b]$p = 0.020$

The 24-hour urinary excretion of 8-iso-$PGF_{2\alpha}$ and the ratio of 8-iso-$PGF_{2\alpha}$ to GFR are shown in Table II by diabetes status in smokers and non-smokers. The daily urinary excretion of 8-iso-$PGF_{2\alpha}$ (and also the value of 8-iso-$PGF_{2\alpha}$/GFR) was statistically significantly higher in the group of diabetic subjects in comparison to control subjects. When only non-smoking diabetic subjects and non-smoking control subjects were compared the difference in the urinary excretion of 8-iso-$PGF_{2\alpha}$ remained statistically significant. Among the diabetic subjects there was no difference between smokers and non-smokers. Although diabetic smokers had higher values than non-diabetic smokers, this difference was not statistically significant. Smoking seemed to be associated with higher excretion of 8-iso-$PGF_{2\alpha}$ among control subjects, but, again, this association was not statistically significant.

Table III shows the 24-hour urinary excretion of 8-iso-$PGF_{2\alpha}$ and the 8-iso-$PGF_{2\alpha}$/GFR values among non-smokers classified by PON genotype and diabetes status. When all non-smoking study subjects were analyzed together regardless of diabetes status, the LL homozygotes had a statistically significantly higher 24-hour excretion of 8-iso-$PGF_{2\alpha}$ in comparison with M-allele

carriers (4788.5 ± 4242.9 vs. 2929.6 ± 3033.6 ng/24-hour, p = 0.023, ANCOVA; age, BMI and gender as covariates). With regard to the diabetic subjects there was no significant difference between PON genotypes. In control subjects, the 24-hour excretion of 8-iso-$PGF_{2\alpha}$ was statistically significantly higher among LL homozygotes compared to M-allele carriers and this difference remained significant after adjustment for GFR. If the HDL concentration was added to the covariates in the ANCOVA model (age, BMI, gender and HDL as covariates), the associations between PON genotype and 24-hour 8-iso-$PGF_{2\alpha}$ (p = 0.014) and 8-iso-$PGF_{2\alpha}$/GFR (p = 0.016) remained statistically significant in the group of control non-smokers.

DISCUSSION

The new finding in our study was that PON is associated with the excretion of 8-iso-$PGF_{2\alpha}$. PON LL homozygotes had a higher excretion of 24-hour 8-iso-$PGF_{2\alpha}$ than the M-allele carriers and this association persisted after correction for GFR. Since PON is an antioxidative enzyme bound to HDL particles, this result suggests that PON may contribute to the destruction of some lipid peroxides, which lead to the formation 8-iso-$PGF_{2\alpha}$. Our study implies that PON in persons with the LL genotype may be the least protective against this kind of lipid oxidation. Ultimately, our finding suggest that subjects with the LL genotype are at bigger risk for atherosclerosis than the carriers of the M-allele.

This observation is somewhat confusing, since it was originally assumed that subjects with high PON activity (LL homozygotes) towards paraoxon are better protected against atherosclerotic diseases compared to subjects with low PON activity.[22,23] However, HDL from subjects with the MM (low active) genotype were later found to protect LDL more effectively against peroxidation than the HDL from subjects with the ML or LL genotype.[2] The protective effect of HDL was, therefore, found not to be associated with the absolute levels of HDL cholesterol but rather with the abundance of HDL particles, which contain protective enzymes relative to the concentration of oxidized LDL. The determinants of this ratio are both genetic and environmental. Our observation is thus in line with the current knowledge, according to which PON can scavange the multioxidized phospholipid-containing trienes[24] and that the PON with the LL genotype is the least effective in this action. A limitation of our study was the lack frozen serum samples and, due to this, we were not able to measure PON concentration and activity to test the effect of genotype on these parameters. The interpretation of these measurements seems to be unclear because, as indicated, the high activity of PON against paraoxon can not be interpreted directly as being protective against atherosclerosis.

The lack of an association between the M/L55 genotype and the excretion of 8-iso-$PGF_{2\alpha}$ among diabetic subjects is probably due to the circumstance that the diabetes itself causes oxidative stress which obscures the effect of genetic variation. This hypothesis is in line with our finding that the non-smoking diabetic subjects had statistically significantly higher excretion of 8-iso-$PGF_{2\alpha}$ than non-smoking control subjects. Smoking is also known to increase oxidative stress. In our study, the excretion of 8-iso-$PGF_{2\alpha}$ was higher in non-diabetic smokers than in non-smokers but, maybe due to a small group size among smokers, the difference was not statistically significant. These findings support the theory that systemic oxidative stress is enhanced in subjects with type 2 diabetes and among smokers.

There is apparently an unmeasured substrate for PON whose product modulates atherogenesis. Combining the current knowledge of PON and 8-iso-$PGF_{2\alpha}$, it is tempting to speculate that some metabolite derived from oxidized arachidonic acid finally leading to the formation of 8-iso-$PGF_{2\alpha}$ is, in fact, one of the physiological

substrates of PON. Today, the strongest candidate seems to be one of the modified LDL phospholipids, an oxidized arachidonic acid derivate.[24] These modified arachidonic acid-containing phospholipids occur in minimally modified LDL particles which can activate endothelial cells to bind monocytes, initiate their entry into the vessels walls and promote cellular interactions that are characteristic of the early stages of atherogenesis.[25] This sequence of events is blocked by treatment of modified LDL with purified PON.[24] The oxidized arachidonic acid-containing phospholipids are not well characterized and it is possible that both phospholipid containing oxygenated and cyclized arachidonic acids as well as several fragmentation products of arachidonic acid may serve as substrates for PON.

Two previous studies addressed the association between M/L55 polymorphism and CHD. In a retrospective case-control study there was no association,[26] while a cross-sectional study suggested that the L-allele increases the CHD risk in patients with type 2 diabetes mellitus.[27] The LL genotype was also found to be a predictor of carotid atherosclerosis.[28] These findings are consistent with the results in our study.

In summary, this study confirms some previous findings showing an increased excretion of an oxidative stress marker 8-iso-$PGF_{2\alpha}$ in type 2 diabetes mellitus. We found also an association between the PON M/L55 genotype and urinary excretion of 8-iso-$PGF_{2\alpha}$. The urinary excretion of 8-iso-$PGF_{2\alpha}$ was increased in non-diabetic non-smoking LL homozygotes compared to carriers of the M-allele. Our results support the theory that PON in HDL may protect against oxidative stress by destroying some biologically active lipids.

Acknowledgements

This work was supported by grants from the Medical Research Fund of the Tampere University Hospital and from the Finnish Foundation of Cardiovascular Research.

References

[1] M.I. Mackness, C.A. Abbott, S. Arrol and P.N. Durrington (1993) The role of high-density and lipid soluble antioxidant vitamins in inhibiting low-density lipoprotein oxidation. *Biochemical Journal*, 294, 829–835.

[2] B. Mackness, M.I. Mackness, S. Arrol, W. Turkie and P.N. Durrington (1998) Effect of the human serum paraoxonase 55 and 192 genetic polymorphisms on the protection by high density lipoprotein against low density lipoprotein oxidative modification. *FEBS Letters*, 423, 57–60.

[3] H.G. Davies, R.J. Richter, M. Keifer, C.A. Broomfield, J. Sowalla and C.E. Furlong (1996) The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. *Nature Genetics*, 14, 334–336.

[4] J.D. Morrow, J.A. Awad, H.J. Boss, I.A. Blair and L.J.I. Roberts (1992) Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. *Proceedings of the National Academy of Sciences of the USA*, 89, 10721–10725.

[5] J.D. Morrow, K.E. Hill, R.F. Burk, T.M. Nammour, K.F. Badr and L.J.I. Roberts (1990) A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proceedings of the National Academy of Sciences of the USA*, 87, 9383–9387.

[6] J.D. Morrow, T.A. Minton, K.F. Badr and L.J.I. Roberts (1994) Evidence that the F2-isoprostane, 8-epi-prostaglandin F2alpha, is formed in vivo. *Biochimica et Biophysica Acta*, 1210, 244–248.

[7] K. Takahashi, T.M. Nammour, M. Fukunaga, J. Ebert, J.D. Morrow, L.J.I. Roberts, R. L. Hoover and K.F. Badr (1992) Glomerular actions of a free radical-generated novel prostaglandin, 8-epi-prostaglandin F2alpha, in the rat: evidence for interaction with thromboxane A2 receptors. *Journal of Clinical Investigation*, 90, 136–141.

[8] K.H. Kang, J.D. Morrow, L.J.I. Roberts, J.H. Newman and M. Banerjee (1993) Airway and vascular effects of 8-epi-prostaglandin F2 alpha in isolated perfused rat lung. *Journal of Applied Physiology*, 74, 460–465.

[9] J.D. Morrow, T.A. Minton and L.J. Roberts (1992) The F2-isoprostane, 8-epi-prostaglandin F2 alpha, a potent agonist of the vascular thromboxane/endoperoxide receptor, is a platelet thromboxane/endopeoxidase receptor antagonist. *Prostaglandins*, 44, 155–163.

[10] J.D. Morrow, B. Frei, A.W. Longmire, J.M. Caziano, S.M. Lynch, Y. Shyr, W.E. Strauss, J.A. Oates and L.J.I. Roberts (1995) Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers: smoking as a cause of oxidative damage. *New England Journal of Medicine*, 332, 1198–1203.

[11] J.D. Morrow, K.P. Moore, J.A. Awad, M.D. Ravenscraft, G. Marini, K.F. Badr, R. Williams and L.J.I. Roberts (1993) Marked overproduction of non-cyclooxygenase derived prostanoids (F2-isoprostanes) in the hepatorenal syndrome. *Journal of Lipid Mediation*, 6, 417–420.

[12] N.K. Gopaul, E.E. Änggård, A.I. Mallet, D.J. Betteridge, C. Wolff and J. Nourooz-Zadeh (1995) Plasma 8-epi-PGF2alpha levels are elevated in individuals with non-insulin dependent diabetes mellitus. *FEBS Letters*, 368, 225–229.

[13] F. Catella, M.P. Reilly, N. Delanty, J.A. Lawson, N. Moran, E. Meagher and G.A. FitzGerald (1995) Physio-

logical formation of 8-epi-$PGF_{2\alpha}$ in vivo is not affected by cyclooxygenase inhibition. *Advances in Prostaglandin, Tromboxane, and Leukotriene Research*, 23, 233–236.

[14] O.R. Wirta and A.I. Pasternack (1995) Glomerular filtration rate and kidney size in type 2 (non-insulin-dependent) diabetes mellitus. *Clinical Nephrology*, 44, 1–7.

[15] W.M. Kirkendall, M. Feinleib, E.D. Freis and A.L. Mark (1980) Recommendations for human blood pressure determination by sphygmomanometers. *Circulation*, 62, 1146.

[16] W.T. Friedewald, R.I. Levy and D.S. Fredrickson (1972) Estimation of the concentration of low-density-lipoprotein cholesterol in plasma, without use of the preparative ultrasentrifuge. *Clinical Chemistry*, 18, 499–502.

[17] E.S. Garnett, V. Parsons and N. Veall (1967) Measurement of glomerular filtration-rate in man using a 51Cr-edetic-acid complex. *Lancet*, 15, 818–819.

[18] B. Frei, R. Stocker and B.N. Ames (1988) Antioxidant defences and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences of the USA*, 88, 9748–9752.

[19] G. Catignani and J. Bieri (1983) Simultaneous determination of retinol and alpha-tocopherol in serum or plasma by liquid chromatography. *Clinical Chemistry*, 29, 708–712.

[20] G. Ellman (1993) Tissue sulphydryl groups. *Archives of Biochemistry and Biophysics*, 82, 70–77.

[21] R. Humbert, D.A. Adler, C.M. Disteche, C. Hassett, C.J. Omiecinski and C.E. Furlong (1993) The molecular basis of the human serum paraoxonase activity polymorphism. *Nature Genetics*, 3, 73–76.

[22] J. McElveen, M.I. Mackness, C.M. Colley, T. Peard, S. Warner and C.H. Walker (1986) Distribution of paraoxon hydrolytic activity in the serum of patients after myocardial infarction. *Clinical Chemistry*, 32, 671–673.

[23] S. Secchiero, M. Mussap, M. Zaninotto, R. Bertorelle and A. Burlina (1989) Serum arylesterase (paraoxonase) activity following myocardial infarction. *Clinica Chimica Acta*, 183, 71–75.

[24] A.D. Watson, J.A. Berliner, S.Y. Hama, B.N. La Du, K.F. Faull, A.M. Fogelman and M. Navab (1995) Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *Journal of Clinical Investigation*, 96, 2882–2891.

[25] A.D. Watson, N. Leitinger, M. Navab, K.F. Faull, S. Horkko, J.L. Witztum, W. Palinski, D. Schwenke, R.G. Salomon, W. Sha, G. Subbanagounder, A.M. Fogelman and J.A. Berliner (1997) Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *Journal of Biological Chemistry*, 23, 13597–13607.

[26] T. Zama, M. Murata, Y. Matsubara, K. Kawano, N. Aoki, H. Yoshino, G. Watanabe, K. Ishikawa and Y. Ikeda (1997) A 192Arg variant of the human paraoxonase (HUMPONA) gene polymorphism is associated with an increased risk for coronary artery disease in the Japanese. *Arteriosclerosis, Thrombosis and Vascular Biology*, 17, 3565–3569.

[27] M.C. Garin, R.W. James, P. Dussoix, H. Blanche, P. Passa, P. Froguel and J. Ruiz (1997) Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. *Journal of Clinical Investigation*, 99, 62–66.

[28] H. Schmidt, R. Schmidt, K. Niederkorn, A. Gradert, M. Schumacher, N. Watzinger, H.P. Hartung and G.M. Kostner (1998) Paraoxonase PON1 polymorphism leu-Met54 is associated with carotid atherosclerosis: results of the Austrian Stroke Prevention Study. *Stroke*, 29, 2043–2048.